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"UnPAKing" Human Immunodeficiency Virus (HIV) Replication: Using Small Interfering RNA Screening To Identify Novel Cofactors and Elucidate the Role of Group I PAKs in HIV Infection

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In order to identify novel proviral host factors involved in human immunodeficiency virus (HIV) infection, we performed a screen of a small interfering RNA (siRNA) library targeting 5,000 genes with the highest potential for being targets for therapeutics. Many siRNAs in the library against known host factors, such as TSG101, furin, and CXCR4, were identified as inhibitors by the screen and thus served as internal validation. In addition, many novel factors whose knockdown inhibited infection were identified, including Pak3, a member of the serine/threonine group I PAK kinases. The HIV accessory factor Nef has been shown to associate with a PAK kinase, leading to enhanced viral production; however, the exact identity of the kinase has remained controversial. Prompted by the Pak3 screen hit, we further investigated the involvement of group I PAK kinases in HIV using siRNA. Contrary to the current literature, Pak1 depletion strongly inhibited HIV infection in multiple cell systems and decreased levels of integrated provirus, while Pak2 depletion showed no effect. Overexpression of a constitutively active Pak1 mutant also enhanced HIV infection, further supporting its role as the dominant PAK involved.

Despite large advances in treatment over the past two decades, human immunodeficiency virus (HIV)/AIDS continues to be a global health threat. The significant decline in AIDS deaths seen with the advent of antiretroviral therapy in the developed world is now becoming more gradual (36) as the incidence of drug resistance increases. The strategy for HIV drug design has been to target the viral proteins, namely reverse transcriptase, integrase, protease, and gp41, in order to maximize antiviral effect and minimize potential host toxicity (29). However, the low fidelity of genomic copying mediated by reverse transcriptase leads to mutations in the viral genes that render them resistant to antiviral drugs (20, 28). Issues of resistance and lack of an effective vaccine have recently led to the targeting of host factors for antiviral drug development (30). Because of the small size of the viral genome, HIV relies heavily on the host cell machinery to complete its life cycle. The first efforts at targeting host factors for antiviral therapy have focused on the receptor (CD4) and coreceptor (CCR5 and CXCR4) (35). Both compound and antibody antagonists against CCR5 are in clinical trials; however, there is concern that treatment with these agents alone may drive the virus to evolve more quickly to CXCR4 usage, a switch generally thought to accelerate disease (24). Recombinant CD4 and antibodies targeting CD4 are also in clinical trials, and it remains to be seen what effects these agents have on the immune system itself. Cyclin-dependent kinase inhibitors, especially those targeting CDK9, have shown in vitro efficacy against HIV infection in multiple systems (30). They have also shown promise in models of HIV-associated nephropathy, highlighting the need to evaluate host cell factors both in primary infection and in HIV-associated secondary effects (30). Other host factors that have been identified as being involved in the HIV life cycle, such as the proviral TSG101 (12) and antiviral APOBEC3G (17), have also been considered as possible therapeutic targets. Although we know about several critical host factors involved in HIV infection, there are many more that remain undiscovered. Considering the issues with the current drug regimens and lack of an effective vaccine, it is critical to find novel targets for HIV therapeutics.

In order to find novel host factors involved in HIV infection, we performed a subgenomic screen of a small interfering RNA (siRNA) library targeting 5,000 genes with the highest potential for being therapeutic targets in HeLaCD4βgal cells challenged with HIV type IIIb (HIV-IIIb). The screen identified several host factors known to be involved in multiple stages of the viral life cycle, serving as a validation of the screen design. We also identified the group I p21-activated kinase Pak3 as a novel cellular factor whose knockdown had a negative impact on infection. Studies have shown that immunoprecipitation of Nef from cells also precipitates a member of the group I family of p21-activated kinases (PAKs) whose activation leads to enhancement of viral transcription from the long terminal repeat (LTR) (27, 34, 40). While the discovery that this Nef-associated kinase (NAK) was a group I PAK was unequivocal, the exact PAK family member involved has remained controversial, with the majority of the literature in support of Pak2. This led us to further investigate the role of the three group I PAKs in HIV infection using siRNA technology. Contrary to previous studies, we found that inhibition of Pak1 and Pak3 expression using siRNA inhibited HIV-IIIb, while PAK2 depletion had no effect. Studies in Jurkat T cells challenged with HIV-IIIb and U373-Magi-CCR5E cells challenged with HIV-BaL continued to suggest a role for PAK1, while PAK2 depletion had no effect. Knockdown of Pak1 or Pak3 in HeLaCD4βgal

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cells resulted in decreased integrated HIV provirus, indicating a block at early stages of infection. In support of the siRNA data, overexpression of constitutively active Pak1 enhanced HIV infectivity. Together, these studies implicate Pak1 as being the dominant PAK involved in HIV infection and argue against a significant role for Pak2.

MATERIALS AND METHODS

siRNA. GL2 luciferase(catalog no. D-001100-01-20), GL3 luciferase (catalog no. D-001400-01-05), Pak1-3 individual siGENOME duplex (catalog no. D-003521-03), PAK2 siGENOME SMARTpool (catalog no. M-003597-02), and Pak3-3 individual siGENOME duplex (catalog no. D-003614-03) siRNAs were obtained from Dharmacon. In the case of GL2 siRNA, additional amounts of the same sequence were also obtained from QIAGEN. Two siRNAs against Tat were synthesized and pooled for use as a positive control for inhibition of HIV infection: (i) CUGCUUGUACCAAUUGCUA-d(TT), which has been previously published (11), and (ii) GCCUUAGGCAUCUCCUAUG-d(TT), which was designed using the Dharmacon siDESIGN center. Pak1-0 siRNA with the sequence AGAGCUGCUACAGCAUCAA-d(TT) was previously reported (2) and synthesized by QIAGEN. Pak3-2 siRNA with the sequence CCAGGUGAU CCAUAGAGAU-d(TT) was designed using the Dharmacon siDESIGN center and synthesized by QIAGEN.

Cell lines and maintenance. HeLaCD4βgal and U373-Magi-CCR5E cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH, from Michael Emerman (22) and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1× Pen/Strep/glutamine, 0.2 mg/ml Geneticin, and 0.1 mg/ml Hygromycin B (Invitrogen). Jurkat cells were maintained in RPMI supplemented with 10% FBS and 1× Pen/Strep/glutamine (Invitrogen).

siRNA screening in HeLaCD4βgal cells. High-throughput siRNA retro-transfection of HeLaCD4βgal cells was carried out essentially as described elsewhere (2). Briefly, individual siRNAs of a subgenomic library focused against 5,000 potential drug targets (collection details can be found at http://function.gnf.org) and control GL2 and Tat siRNAs were spotted at 14 ng/well in white opaque 384-well plates (Greiner) containing one siRNA sequence per well from the library. A solution of 2% oligofectamine (Invitrogen) in serum-free Opti-MEM medium (Invitrogen) was added to each well (10 μl), and complexes were allowed to form for 15 to 20 min. All 384-well dispenses were done using a Multidrop apparatus (Titertek). HeLaCD4βgal cells (1,000 cells/30 μl/well in serum-free Opti-MEM) were then added, and the plates were incubated overnight, followed by the addition of 20 μl of 30% FBS–DMEM with 200 ng/ml of HIV-IIIb (Advanced Biotechnologies Inc.). After 72 h, infection was assessed by measuring β-galactosidase production using Gal Screen (Applied Biosystems). All 384-well plate reading was done using the CLIPR apparatus (Molecular Devices). The entire library was run in duplicate. Data for each siRNA were compared to the mean signal of the entire plate and expressed as the ratio AFA/MFA, which is the average fold activation (AFA) divided by the adjusted standard deviation of the fold activation (MFA). The MFA penalizes the value for fold activation if the standard deviation between the replicates is high. For those ratios less than one, the value was converted to a negative number to represent fold inhibition.

Follow-up experiments run in HeLaCD4βgal or U373-Magi-CCR5E cells were conducted by the same method used for screening; however, 12 replicates were run per 384-well plate and the data were expressed as percent inhibition compared to the negative control GL2 siRNA. Cytotoxicity of the siRNA was measured at 96 h posttransfection by adding an equal volume of a 1:4 dilution of Cell Titer Glo (Promega) and reading luminescence, with the data again expressed as a percent inhibition compared to GL2.

siRNA validation by Western blotting. siRNA (800 ng) was spotted in 250 μl of serum-free Opti-MEM in six-well plates followed by the addition of 250 μl of 1.5% Lipofectamine 2000 (Invitrogen) in serum-free Opti-MEM. Plates were incubated at room temperature for 20 min to allow for complex formation. HeLaCD4βgal or U373-Magi-CCR5E cells (3×10^5 in 1.5 ml of serum-free Opti-MEM) were then added and incubated overnight followed by the addition of 1 ml of 30% FBS–DMEM. Cells were harvested 72 h posttransfection by scraping in phosphate-buffered saline (PBS) and lysed in cell lysis buffer (20 mM HEPES, pH 7.2–10 mM KCl–1 mM EDTA–1% Triton X-100–1× protease inhibitors; Sigma Chemical Co.) for 1 hour on ice. Total protein concentration of the lysates was measured using the Micro-BCA kit (Promega), and equal protein amounts were loaded onto 4-to-12% NuPage bis-Tris gel (Invitrogen) and subjected to electrophoresis as suggested by the manufacturer. Following transfer to nitrocellulose, blots were blocked with 5% nonfat milk in PBST (phosphate-buffered saline with 0.05% Tween 20) and then subjected to immunoblotting with the following antibodies: rabbit polyclonal anti-Pak1, -Pak2, and -Pak3 antibodies from Cell Signaling Technologies, goat anti-tubulin antibody from Santa Cruz Biotechnology, horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody from Sigma Chemical Co., and HRP-conjugated donkey anti-goat secondary antibody from Promega. All antibodies were used at dilutions suggested by the manufacturer and were diluted in 5% nonfat milk in PBST. Bands were visualized using the ECL-plus detection reagent (Amersham).

siRNA transfection of Jurkat T cells. Jurkat T cells were washed once in PBS and resuspended in serum-free Opti-MEM (Invitrogen) at high density (2.4×10^8/ml), and 50 μl was added to 1 nmol of siRNA (50 μl of 20 μM) in a 0.2-cm gap cuvette. The mixture was subjected to electroporation using the Bio-Rad Gene Pulser Xcell module under conditions suggested by the manufacturer (140 V, 1,000 μF, exponential decay) and then transferred to 12 ml of RPMI supplemented with 10% FBS without antibiotics for 24 h. Cells were then pelleted, and viable cells were counted by trypan blue exclusion and resuspended at a density of 1.7×10^6/ml in RPMI supplemented with 10% FBS and 1× Pen/Strep/glutamine. For infection studies, 300 μl of siRNA-treated cells was added to wells of a 48-well plate, and either 2 ng or 0.5 ng of HIV-IIIb, corresponding to multiplicities of infection of 0.0005 and 0.000125, respectively, based on viral titer provided by the manufacturer, was added to each well. After three additional days, the cells were harvested, washed three times in PBS, and lysed, and infection was measured by p24 enzyme-linked immunosorbent assay of the cell lysates. For cytotoxicity, 300 μl of siRNA-treated cells was added to wells of a 48-well plate and after 3 days, cell viability was measured using Cell Titer Glo (Promega) or Alamar Blue (TREK systems). To determine siRNA efficacy, cell lysates were prepared and analyzed as in siRNA validation studies at 72 h postelectroporation.

Effects of Pak1 and Pak3 overexpression in HeLaCD4βgal cells. Constructs expressing Pak1 or a constitutively active mutant (T423E) of Pak1 (Pak1CA) were generously provided by Ken Nomoto. The open reading frame of Pak1 was cloned by reverse transcription-PCR (RT-PCR) from human fetal heart total RNA (primers: sense, 5′-CACCATGTCAAATAACGGCCTAGACATTCA AGAC-3′; antisense, 5′-GTGATTGTTCTTTGTTGCCTCCTTAGCTGC-3′) and cloned into pCDNA3.1D/V5-His/Topo (Invitrogen). Constitutive active mutant PAK1-T423E was produced with the QuikChange2 mutagenesis kit (Stratagene). The construct expressing wild-type Pak3 was generously provided by R. Cerrione, Cornell University. cDNA retro-transfection of HeLaCD4βgal cells was conducted as described previously (8, 19), but in a lower-throughput format. Briefly, 1.28 μg of Pak1 or Pak1CA, Pak3, or pcDNA3.1(-) DNA, along with 320 ng of an HIV LTR-luciferase construct (derived by PCR amplification of the LTR sequence of strain HxB2 that was directly cloned into pGL-Basic vector from Promega) were spotted in 32 μl of serum-free Opti-MEM in 12-well plates. A solution of 1% Gene Juice (Novagen) in serum-free Opti-MEM was then added to each well (320 μl), and complexes were allowed to form for 10 to 15 min at room temperature. HeLaCD4βgal cells in DMEM–10% FBS at 6×10^4/ml (1 ml per well) were then added. After overnight incubation, 90 ng of HIV-IIIb was added to each well and the plates were cultured for three additional days before measuring infection using Brite Glo (Promega) and reading on the CLIPR apparatus (Molecular Devices). For assessment of protein expression, parallel uninfected wells were harvested at 72 h posttransfection and processed for Western blot analysis as described for siRNA validation.

Effects of group I PAK siRNA on integrated HIV provirus. Levels of integrated HIV provirus in HeLaCD4βgal cells transfected with control GL2 siRNA or siRNA against Tat, Pak1, Pak2, or Pak3 were determined by a nested PCR method based on those previously described (7). Briefly, cells were transfected with siRNA in six-well dishes as outlined above for Western blot analysis and then exposed to 180 ng p24 of HIV-IIIb (in the presence of efavirenz for the GL2 EFV sample) for 2 hours. Cells were then washed twice with PBS to remove unadsorbed virus and either harvested by trypsinization (time zero) or resuspended in medium for 24 h followed by harvest by trypsinization ($t = 24$). Total DNA was isolated from the harvested cells using the DNeasy kit (QIAGEN), and 100 ng of total DNA was then used for the first-step PCR using the following primers (described in reference 7) at 200 nM each in a 50-μl volume: Alu, 5′-CTCACGCCTGTAATCCCAGCA-3′; MH532, 5′-GAGTCCTGCGTCGAG AGAGC-3′. The first-step PCRs used an annealing temperature of 55°C and an elongation time of 3 min for 20 cycles. Following the first PCR step, 1 μl of each reaction mixture was used in a subsequent Taqman analysis using the following primers at 300 nM each and probe at 100 nM in a 20-μl reaction volume: MH531, 5′-TGTGTGCCCGTCTGTTGTGT-3′; MH532 (described above); and late RT probe, 5′-carboxyfluorescein-CAGTGGCGCCCGAACAGGGA-tetramethyl

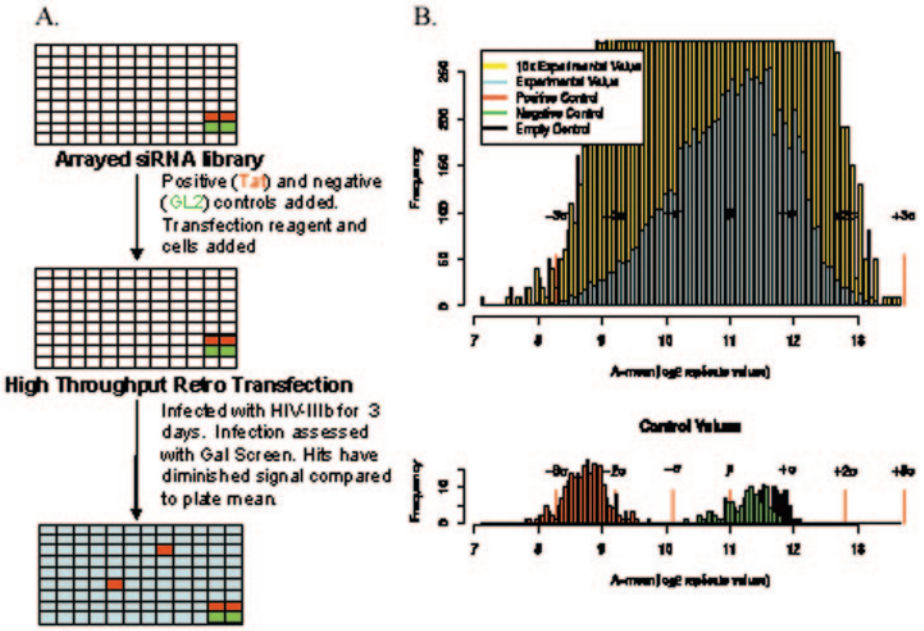

FIG. 1. High-throughput siRNA screen layout and performance of controls. (A) Negative control (GL2; green boxes) and positive inhibition control (Tat; red boxes) siRNAs were spotted into the wells of a 384-well opaque plate containing one sequence of library siRNA/well followed by the addition of transfection reagent. After complex formation, HeLaCD4βgal indicator cells were added and incubated overnight, after which HIV-IIIb (4 ng/well) was added. Three days later, levels of infection were assessed by measuring β-galactosidase produced from the Tat-responsive LTR–β-Gal reporter within the cells, using Gal Screen. Hits give diminished signal compared to the plate mean, much like the control Tat siRNA. (B) Performance of all siRNAs in the library (blue bars) showed a Gaussian distribution around the mean signal for the entire screen. Negative control siRNA (GL2; green bars) performed at or just above the mean, overlapping with empty wells (black bars), and positive control siRNA (Tat; red bars) inhibited infection with signals 2 to 3 standard deviations below the mean.

carboxyrhodamine-3′. All primers/probes were described previously (7). Taqman analysis was performed on an ABI Prism 7900HT (Applied Biosystems). Each reaction contained 1× Taqman universal master mix (Applied Biosystems) and was run in a 384-well plate using the standard parameters defined by the system. With each Taqman experiment, a plasmid containing the LTR and psi sequence of HIV was used to generate a standard curve for relative quantitation. To control for loading, rRNA levels were also measured and used for normalization using an rRNA control primer/probe set (Applied Biosystems).

RESULTS

siRNA screening of a focused library identified PAK3 as being involved in HIV infection of HeLaCD4βgal cells. In order to find novel proviral host factors involved in HIV infection, we performed a subgenomic screen of an siRNA library targeting 5,000 genes with the highest potential for being therapeutic targets in HeLaCD4βgal indicator cells challenged with HIV-IIIb. Details of the library design and genes targeted can be found at http://function.gnf.org. Each gene is represented by two nonoverlapping siRNA sequences of variable efficacy, resulting in a total of 10,000 siRNAs screened. The cells were transfected using a high-throughput reverse transfection protocol previously described (2, 8). Negative control (GL2) and positive control (Tat) siRNAs were spotted into wells of 384-well plates containing the library siRNA (one sequence per well) followed by the addition of transfection reagent and HeLaCD4βgal cells (Fig. 1A). After 24 h, each well was infected with HIV-IIIb. Infection was allowed to proceed for 3 days to allow for effects on all stages of infection from entry to release and spread throughout the culture to be observed. Infection was then assessed by measuring the amount of β-galactosidase produced via the viral LTR promoter using a chemiluminescent substrate (Gal Screen). The distribution of signals from all of the siRNAs and controls is shown in Fig. 1B, where the positive control Tat siRNA showed inhibition between 2 and 3 standard deviations from the mean and negative control GL2 siRNA performed close to the mean. The entire screen was conducted in duplicate, and the data are expressed as a ratio of the AFA and MFA, a value that takes into account both the effect of each siRNA and the deviation between the replicates. For those genes whose AFA was less than 1 (inhibitors of infection), values were converted to negative fold of activation. Many siRNAs against genes already known to be involved in various stages of HIV infection, such as TSG101 (12), furin (38), and CXCR4, were identified as inhibitors by the screen and thus served as internal validation of the screen design (Table 1). For TSG101 and furin, both siRNAs designed to target the protein were effec-

TABLE 1. Performance of siRNA against known factors and Pak3 in siRNA screen

Gene	AFA/MFA[a]
TSG101, 1	−6.61
TSG101, 2	−3.15
Furin, 1	−2.31
Furin, 2	−3.80
CXCR4	−2.75
PAK3	−2.86

[a] Average fold activation (AFA) divided by the adjusted standard deviation of the fold activation (MFA).

tive; however, only one worked well for CXCR4, highlighting the potential differences in efficacy between the two siRNAs designed for each gene in the collection.

In addition to the known factors, many novel factors whose depletion inhibited infection were identified. Within these hits, the kinase Pak3 was identified as being involved in HIV infection (Table 1). Pak3 is a member of the group I PAK kinases, which also includes Pak1 and Pak2. The group I PAKs have been shown to be involved in cytoskeletal organization, cell morphology and motility, neurogenesis, cancer metastasis, and apoptosis. They activate ERK and mitogen-activated protein kinases, although this may depend on the cell type and the stimulus (growth factors, T-cell receptor [TCR] ligation, or integrins) (21, 43). In T cells, both PAK1 and PAK2 can be activated following TCR ligation, leading to the activation of NFaT (41) and JNK pathways (33), and enabling expression from the serum response element (9, 10). Mutations in PAK3 have been linked to X-linked mental retardation, emphasizing its importance in neuronal function (5).

Both Pak1 and Pak2 have been shown to be able to associate with Nef, leading to enhanced viral production (13, 31). While the strongest evidence points to Pak2 as being the main PAK involved in enhancement of infection (23, 31, 32), none of the previously published studies has used siRNA to determine which endogenous PAK kinases are specifically involved in physiologic HIV infection. The identification of Pak3 in this siRNA screen prompted a more in-depth analysis of the role of group I PAK kinases in HIV infection.

Knockdown of endogenous Pak1 and Pak3, but not Pak2, inhibits HIV-IIIb infection of HeLaCD4βgal cells. We obtained siRNAs against Pak1, Pak2, and Pak3 and validated each in HeLaCD4βgal cells by Western blotting (Fig. 2A) (8). Both siRNAs against PAK1 effectively decreased protein levels, with Pak1-0 being more effective than Pak1-3. The validated SMARTpool siRNA against Pak2 (9) was also very effective, with near-total protein knockdown by 72 h posttransfection. Both Pak3 siRNAs were also effective at protein depletion. The siRNAs against group I PAKs were then tested for their abilities to inhibit HIV infection in the original screen assay (Fig. 2B) (8). Pak1-0 was almost as effective at blocking HIV as the Tat siRNA control, inhibiting by ~70%, while Pak1-3 was less effective, blocking by ~38%. The relative efficacies of the Pak1 siRNAs at decreasing protein levels were reflected in their abilities to inhibit HIV infection. Despite having apparent differences in protein knockdown, both siRNAs against Pak3 (Pak3-2 and Pak3-3) blocked infection by 40 to 50%. This suggests that there is a threshold level of Pak3 protein required for efficient viral replication and that even more modest changes can lead to inhibition. Contrary to what is suggested in the current literature, siRNA against PAK2 had little to no effect on HIV infection despite being able to effectively deplete protein. None of the siRNAs tested had significant cytotoxicity in the HeLa cells (Fig. 2B), ruling out overt toxicity as a mechanism for infection inhibition.

Sequence analysis of the regions targeted by these siRNAs showed no overlap of more than 81% and no spans longer than 7 nucleotides matching between the different group I PAKs (data not shown). However, in order to prove that cross-knockdown of other group I PAKs was not occurring, we probed lysates from cells treated with siRNA against Pak2 and Pak3 for Pak1 expression and also probed lysates from cells treated with Pak1 siRNA for Pak2 expression (Fig. 2C). Neither Pak3 siRNAs nor the Pak2 pool affected Pak1 levels, and Pak1 siRNA did not affect Pak2 levels. Together, these results suggest a significant role of Pak1 and Pak3 in HIV infection of HeLaCD4βgal cells and argue against a major role for Pak2.

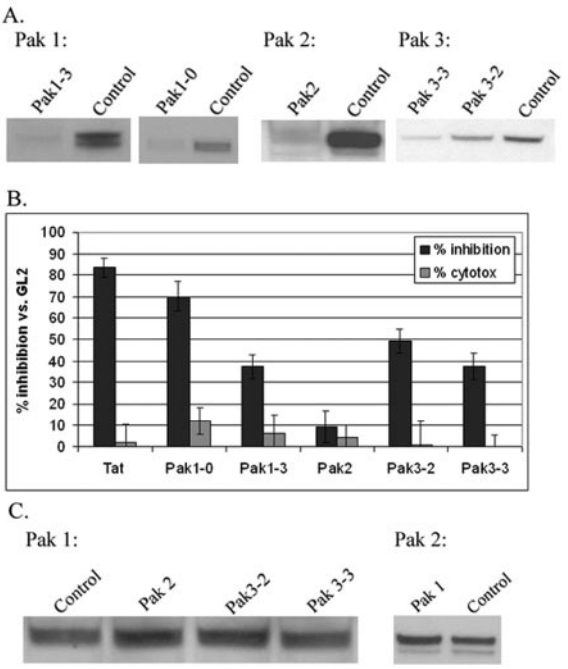

FIG. 2. Efficacy of group I PAK siRNAs in HeLaCD4βgal cells challenged with HIV. (A) Group I PAK siRNAs were transfected into HeLaCD4βgal cells. Cells were harvested after 72 h and immunoblotted to evaluate depletion of the target proteins. Each siRNA tested effectively reduced its target protein. (B) Group I PAK siRNAs were tested for their ability to block HIV-IIIb infection (black bars) and for their cytotoxicity (gray bars) in HeLaCD4βgal cells compared to negative control GL2 siRNA, with Tat siRNA as a positive control. Data shown are the percent inhibition or cytotoxicity compared to the negative control GL2 siRNA and are the summary of at least four replicate assays with 12 replicate wells in each assay. (C) Group I PAK siRNAs were transfected into HeLaCD4βgal cells. After 72 h, cells were harvested and immunoblotted to evaluate knockdown of other group I PAKs. Neither Pak2 nor Pak3 siRNAs decreased levels of Pak1, confirming what was suggested by the overlap analysis (data not shown). Pak1 siRNA also had no effect on levels of Pak2, ruling out cross-reactivity as the mechanism for HIV inhibition.

Pak1, but not Pak2, siRNA blocks HIV-BaL infection in U373-Magi-CCR5E cells. In order to see if the effects of depletion of group I PAKs could be extended to other cell types and virus isolates, we looked at the effects of PAK siRNAs on HIV-BaL infection of U373-Magi-CCR5E cells, a glioma line engineered to express CD4, CCR5, and β-galactosidase under the control of the HIV LTR (37). siRNA transfection and infection assessment can be evaluated in these cells analogous to the HeLaCD4βgal system (8). As was seen in the HeLaCD4βgal cells, siRNA against Pak1 inhibited infection, while siRNA against Pak2 had no effect (Fig. 3B), despite effective protein knockdown (Fig. 3A). The lower efficacy of siRNA Pak1-3 can be explained by its diminished capacity to decrease Pak1 protein levels in the U373 cell line (Fig. 3A).

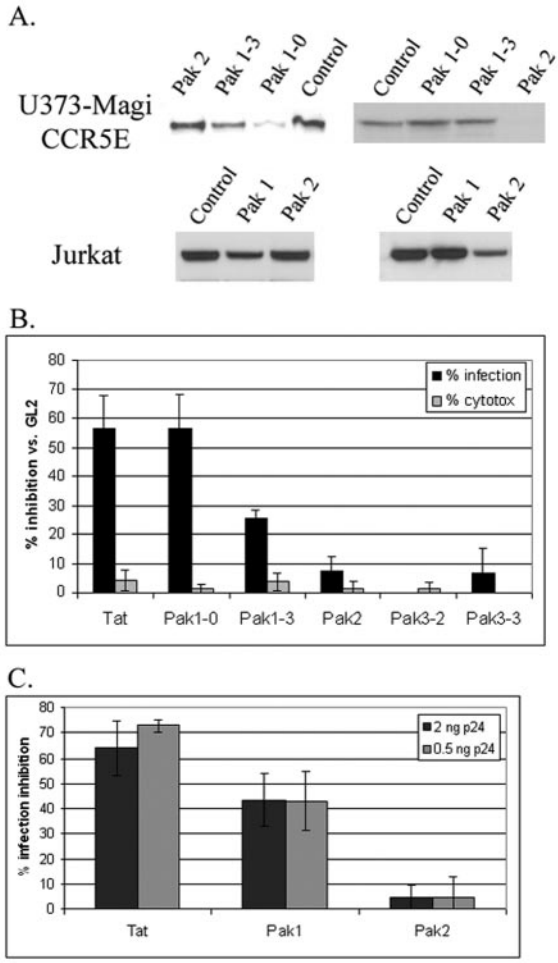

FIG. 3. Efficacy of group I PAK siRNAs in other cell types challenged with HIV. (A) Group I PAK siRNAs were transfected into U373-Magi-CCR5E cells or electroporated into Jurkat cells. Cells were harvested after 72 h and immunoblotted to evaluate depletion of the target proteins. Each siRNA tested effectively reduced its target protein except for Pak3 siRNA in U373-Magi-CCR5E cells, where no decrease in protein levels was observed despite good mRNA depletion (data not shown). (B) Group I PAK siRNAs were tested for their ability to block HIV-IIIb infection (black bars) and for their cytotoxicity (gray bars) in U373-Magi-CCR5E cells compared to the negative control GL2 siRNA, with Tat siRNA as a positive control. Data shown are the percent inhibition or cytotoxicity compared to the negative control GL2 siRNA and are the summary of at least four replicate assays with 12 replicate wells in each assay. (C) The strongest siRNAs against Pak1 and the Pak2 pool were electroporated into Jurkat cells and tested for their abilities to inhibit HIV infection at 2 ng p24 (black bars) or 0.5 ng p24 (gray bars) input. Data shown are the percent inhibition compared to negative control GL2 siRNA and are the summary of at least four replicate assays with two replicates in each assay.

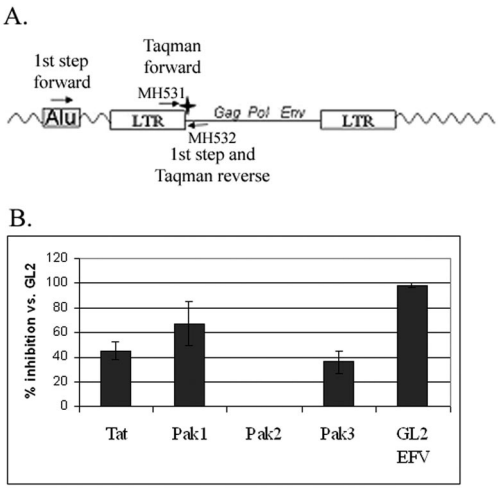

FIG. 4. Effect of group I PAK siRNAs on levels of integrated HIV provirus. (A) Diagram of nested PCR assay for integrated provirus with locations of primers used. Integrated provirus can be amplified using a forward primer specific for Alu sequences and a reverse primer specific for viral sequence in the first-step PCR and then quantified with a virus-specific primer/probe set using Taqman quantitative PCR. All primers/probes were previously described (7). (B) HeLaCD4βgal cells were transfected with negative control GL2 siRNA or siRNA against Tat, Pak1, Pak2, or Pak3 and then infected with HIV-IIIb (in the presence of efavirenz for the GL2 EFV sample). After 24 h, cells were harvested and genomic DNA was isolated and used for the first-step PCR with primers described above. One microliter of product from the first step was then used as template for Taqman quantitative PCR. Integrated provirus was quantitated by comparing to a standard curve generated using a plasmid containing the viral sequences targeted by the primer/probe set. Data shown are the percent inhibition compared to the GL2 negative control and are the summary of three independent assays.

Interestingly, siRNA against Pak3 was not inhibitory in this system. Despite being able to detect specific gene knockdown by quantitative PCR, Pak3 protein levels remained unchanged up to 112 h posttransfection (data not shown), suggesting that the lack of antiviral efficacy is due to lack of protein knockdown. Gene chip analysis of Pak3 expression in human tissues shows that the majority of expression is limited to the brain, uterus, and pancreas (details at http://symatlas.gnf.org). Involvement of Pak3 may thus be limited only to certain cell lines and tissue types.

Pak1, but not Pak2, siRNA blocks HIV infection in Jurkat cells. To determine if the pattern observed for Pak1 and Pak2 would extend to a more physiologic cell target, the siRNAs were tested for efficacy in Jurkat cells. Jurkat cells were electroporated with the most effective siRNA against Pak1 (Pak1-0) or with the Pak2 pool. As seen in the other cell types, PAK1 depletion resulted in inhibition of HIV infection, while Pak2 knockdown had minimal effect even at very low viral input (Fig. 3C) despite the specific knockdown of protein levels (Fig. 3A). The lower level of inhibition in Jurkat cells with Pak1 siRNA compared to HeLaCD4βgal cells is reflected by the lower level of protein knockdown seen (Fig. 2A). As seen for the other cell types, none of the siRNAs had any cytotoxicity (data not shown), again eliminating that as the mechanism for inhibition.

Pak1 and Pak3 siRNA decrease the amount of integrated HIV provirus. In order to get an idea of the stage of infection blocked by Pak1 and Pak3 siRNA in HeLaCD4βgal cells, we looked at the amount of integrated provirus in siRNA-treated cells 24 h after HIV-IIIb infection using a nested PCR method based on those previously described (7, 39). Using a 5′ primer targeting Alu sequences along with the 3′ primer targeting an HIV sequence just downstream of the LTR in the first-step PCR allows amplification of integrated provirus that has randomly inserted into the genome at various distances from Alu

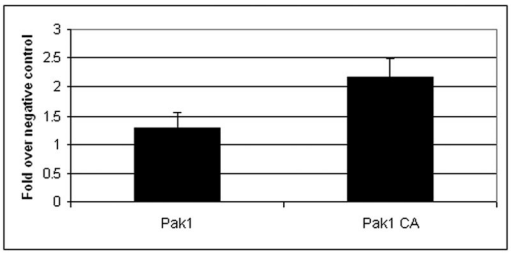

FIG. 5. Effects of Pak1 or Pak1CA on HIV infection. HeLaCD4βgal cells were cotransfected with cDNA encoding wild-type Pak1, Pak1CA, or control plasmid along with a luciferase reporter under the control of the HIV-LTR, followed after 24 h by infection with HIV-IIIb. Infection was assessed after 3 days by measuring luciferase activity. Wild-type Pak1 showed only marginal enhancement of infection compared to control. However, Pak1CA enhanced infection by twofold compared to the control. Data shown are the fold enhancement over negative control plasmid and are the summary of three independent assays with two replicates in each assay.

sequences (Fig. 4A). The relative amount of integrated provirus amplified in the first reaction can then be quantitated by using a primer/probe set specific for a region of the viral sequence from the U5 portion of the LTR to the 5′ end of the gag gene (late RT) using Taqman quantitative PCR. For quantitation, samples were compared to a standard curve generated from a plasmid containing the HIV LTR and sequences upstream of Gag, and rRNA levels were also measured for normalization. As shown in Fig. 4B, knockdown of Pak1 results in significant decreases in the amount of integrated provirus, while Pak2 knockdown has no effect. Pak3 knockdown also leads to a decrease in integrated provirus, with a level of inhibition comparable to that seen for total infection over time. Tat siRNA also partially decreased levels of integrated provirus, a finding consistent with previous reports that siRNA against Tat can degrade incoming viral genomic RNA (11). These results are consistent with previous studies suggesting that PAKs may be involved in early stages of HIV infection (40) but again implicate Pak1 rather than Pak2 as being the main PAK involved.

Overexpression of constitutively active Pak1 enhances HIV-IIIb infectivity. After finding that Pak1 depletion by RNA interference inhibited infection, we tested whether overexpression of wild-type Pak1, wild-type Pak3, or a constitutively active form of Pak1 (Pak1CA) could enhance infection in HeLaCD4βgal cells. All constructs expressed comparable amounts of protein well over endogenous levels (data not shown). Overexpression of wild-type Pak1 had very little effect on infection; however, Pak1CA enhanced HIV-IIIb infection by twofold over the negative control (Fig. 5). Overexpression of wild-type Pak3 also had no effect on HIV infection (data not shown). The reciprocal effect seen for the constitutively active form of Pak1 further strengthens the evidence supporting its role in HIV infection.

DISCUSSION

With the rapid mutation of HIV generating drug-resistant viruses at a rate faster than new drug development and no vaccine against the virus in sight, finding novel targets for HIV treatment is critical. Retroviruses like HIV rely heavily on host cell machinery to complete their life cycle; however, relatively few of the cellular cofactors involved in HIV replication have been discovered. While there are many reports in the literature using yeast two-hybrid screening and microarray analysis to identify new cellular cofactors, little genome-scale screening of siRNA libraries within the context of productive HIV infection have been reported. High-throughput genomic screening allows for the rapid identification of novel host factors involved in infection, while focusing the library on genes with the most potential to be chemically tractable narrows the field to those genes most easily targeted by therapeutics. We identified several factors known to be involved in multiple stages of HIV infection in the screen, including TSG101, CXCR4, and furin, which served as an internal validation of the screen design. We were also able to identify a novel target, Pak3, from the screen, as well as many other potential targets which are currently being investigated. This powerful technique will enable the identification of new potential therapeutic targets for HIV as well as help elucidate more of the basic biology of the virus.

Pak3 itself is a novel cofactor; however, other members of the group I PAK family have already been shown to enhance HIV infection through interactions with the viral Nef protein (27, 34, 40). There was controversy early on as to whether the NAK was Pak1 or Pak2. Fackler et al. identified the NAK as Pak1 (13); however, part of their conclusions were based on recognition by a Pak1 antibody that cross-reacts with Pak2. Not all of the claims made in the article have been directly refuted: a peptide shown by other labs to specifically block Pak1 activity (15) was shown to inhibit HIV infection, a result that has not been challenged in the literature. Two other groups (1, 31) identified the NAK as Pak2, with the strongest evidence being that precipitated NAK was sensitive to caspase-3 cleavage, a feature of Pak2 that highlights its involvement in apoptosis. This evidence in support of Pak2 was strengthened by studies in multiple cell types, including monocytic and T-cell lines, and by the ability to substitute exogenously expressed Pak2 and not Pak1 for NAK activity. Further studies have also indicated that Nef can activate Pak2 by recruitment into lipid rafts (23), where association of Nef with Vav and phosphatidylinositol-3-kinase (PI-3K), the upstream activators of PAK, lead to its activation independent of TCR stimulation. This highlights a mechanism whereby Nef can mimic T-cell stimulation as a means of enhancing viral replication. Even though the majority of current evidence supports Pak2 as being the NAK, most of the studies were done in systems where Nef, Pak1/2, or both were overexpressed followed by immunoprecipitation and in vitro kinase assays. None of the published studies has investigated the effects of knocking down endogenous group I PAKs on HIV infection.

In our studies, siRNA against Pak2 had no effect on HIV infection in any of the systems tested, despite effective protein knockdown. In contrast to what is suggested by the literature, Pak1 depletion had the strongest effect of any of the group I PAKs, blocking infection by up to 75% in long-term assays in multiple cell types and significantly blocking proviral integration. Pak3 showed more moderate inhibition, which depended upon the cell type being studied. These results argue against a role of Pak2 in HIV infection and instead implicate Pak1 as being the dominant PAK involved. Although siRNA against

Pak1 were included in the siRNA collection, they failed to inhibit HIV during the screen. After having the Pak1 siRNA sequences used in the collection resynthesized, they effectively inhibited infection and decreased Pak1 protein levels in HeLaCD4βgal cells (data not shown). Because proper target knockdown is not assessed in parallel while running the screen, a lack of phenotype during the screen cannot be interpreted as a lack of involvement in the pathway. More in-depth studies, such as those reported here, are necessary to rule out transfection or degradation issues that may affect siRNA efficacy during the screen.

Group I PAK kinases have been implicated in a wide variety of cellular processes and signaling cascades. Many of the downstream effectors of PAK activation have been implicated in HIV infection. There are binding sites within the LTR for several cellular transcription factors, including NF-κB, AP-1, and NFaT (14, 26), whose binding leads to activation of viral transcription. Pak1 activates both NF-κB (16) and NFaT (41) and also may activate the Ap-1 pathway through JNK activation (3, 6). Activation of ERK through cytokine signaling pathways involving PAK has been shown to enhance viral protein expression from the LTR through NF-κB and Ap-1 (42). PAK effectors are also involved in HIV infection in ways independent of viral transcription. Inhibitors of p38 mitogen-activated protein kinase, another downstream effector of PAK, have been shown to block HIV replication in vitro partially by preventing virus-mediated apoptosis (25). Phosphorylation of p6gag by active ERK has been implicated in HIV particle formation (18). Viral gp120 itself can activate ERK and actin reorganization, pathways involving PAK activation, in order to mimic the chemotactic response and potentially attract its target cells (4). Our data showing a decrease in the amount of integrated provirus with Pak1 and Pak3 siRNA indicate an early block in proviral replication well upstream of transcriptional effects, which is consistent with results from studies where association of Nef with PAK was disrupted (40). Because of its involvement in cell growth, motility, and activation, it is no surprise that HIV could utilize PAK to potentially enhance multiple stages of the life cycle. The clustering of PAK kinase and its upstream activators through Nef provides the opportunity to activate these downstream effectors, allowing for more efficient viral replication.

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